Urol Res (2010) 38:271–276
DOI 10.1007/s00240-010-0304-3

SYMPOSIUM PAPER

NaPi-IIa interacting proteins and regulation of renal reabsorption of phosphate

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Received: 2 July 2010 / Accepted: 8 July 2010 / Published online: 28 July 2010

Abstract Control of phosphate (P_i) homeostasis is essential for many biologic functions and inappropriate low levels of P_i in plasma have been suggested to associate with several pathological states, including renal stone formation and stone recurrence. P_i homeostasis is achieved mainly by adjusting the renal reabsorption of P_i to the body's requirements. This task is performed to a major extent by the Na/Pi cotransporter NaPi-IIa that is specifically expressed in the brush border membrane of renal proximal tubules. While the presence of tight junctions in epithelial cells prevents the diffusion and mixing of the apical and basolateral components, the location of a protein within a particular membrane subdomain (i.e., the presence of NaPi-IIa at the tip of the apical microvilli) often requires its association with scaffolding elements which directly or indirectly connect the protein with the underlying cellular cytoskeleton. NaPi-IIa interacts with the four members of the Na^+/H^+ exchanger regulatory factor family as well as with the $GABA_A$-receptor associated protein . Here we will discuss the most relevant findings regarding the role of these proteins on the expression and regulation of the cotransporter, as well as the impact that their absence has in P_i homeostasis.

Keywords Renal proximal tubule · NaPi-IIa · NHERF · GABARAP

Proceedings paper from the 3rd International Urolithiasis Research Symposium, Indianapolis, Indiana, USA, 3–4 December 2009.

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Introduction

The need for a proper control of the circulating levels of phosphate (P_i) is illustrated by several pathologies associated with either hypo or hyperphosphatemic states. Thus, hypophosphatemia compromises bone mineralization and may cause cardiac dysfunction, whereas hyperphosphatemia is linked with secondary hyperparathyroidism and reduced life expectancy (for review see [3, 10]). Plasma levels of P_i are kept in the low millimolar range due to the balance between intestinal absorption, bone deposition/bone resorption, and renal reabsorption (for review see [4]). Since P_i is freely filtered in the glomerulus, an efficient renal reabsorption is required to prevent a massive urinary lost of P_i that would otherwise have fatal consequences. In this regard, hypophosphatemia increases intrarenal production of 1,25dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) which in turn leads to higher intestinal absorption of P_i and Ca^{++}, with a concomitant increase in urinary excretion of Ca^{++}. Hypercalciuria is the most prevalent metabolic alteration in renal stone disease and recent studies have suggested that renal reabsorption of P_i is reduced in stone formers and associates with hypercalciuria [17]. Moreover, renal handling of P_i has been proposed as a predictive factor for renal stone recurrence. Among the different hypercalciuric syndromes, hereditary hypophosphatemic rickets with hypercalciuria has been recently shown to associate with mutations in one of the transporters involved in renal reabsorption of P_i (for review see [3, 10, 28]).

Renal reabsorption of phosphate

Reabsorption of P_i from the primary urine takes place preferentially along the proximal tubule with very little

contribution of the distal segments. Several Na-dependent P_i cotransporters from the SLC34 and SLC20 families of solute carriers are expressed in the brush border membrane (BBM) of proximal tubular cells: namely NaPi-IIa (SLC34A1) [8], NaPi-IIc (SLC34A3) [25], and PiT2 (SLC20A2) [30]. NaPi-IIa deficient mice are hypophosphatemic due to an increased urinary excretion of P_i despite a heavy upregulation of NaPi-IIc [2]. These findings, together with the lack of phenotype of NaPi-IIc deficient mice regarding P_i balance [26], indicate that NaPi-IIa is the major cotransporter in the murine kidney. Ablation of both NaPi-IIa [7] and NaPi-IIc [26] results in hypercalcemia and hypercalciuria; as mentioned above, these changes are probably due to a $1,25(OH)_2D_3$-induced upregulation of intestinal Ca^{++} absorption. While disregulation of Ca^{++} homeostasis results in the formation of renal stones in NaPi-IIa deficient mice [7], the absence of NaPi-IIc does not seem to increase renal mineral deposition [26]. In humans, mutations of NaPi-IIa have been reported in patients with hypophosphatemic syndromes [21]; however, it is not clear whether these mutations alone can account for the hypophosphatemic state [31]. Instead, mutations in NaPi-IIc have been identified by several laboratories in a large number of patients with hereditary hypophosphatemic rickets with hypercalciuria (for review see [3, 10]). It remains to be shown whether this different relative contribution of both cotransporters to overall P_i homeostasis in mouse and human represents a species-specific phenomena or responds to yet unknown causes.

Within the murine renal proximal tubule NaPi-IIa is expressed preferentially in the S1 segments and decreases progressively toward the S2 and S3 portions [8]. Under normal dietary conditions it is more abundant in juxtamedular than in superficial nephrons. Its expression in the proximal BBM is under the control of several hormones [parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23)] and metabolic factors ($1,25(OH)_2D_3$, dietary P_i) that regulate renal reabsorption of P_i (for review see [4]). With few exceptions, factors that increase renal reabsorption of P_i (P_i deprivation) do so by upregulating the apical expression of NaPi-IIa, whereas factors that reduce renal reabsorption (PTH, FGF23 or high dietary P_i) lead to downregulation of the cotransporter. Thus, understanding the molecular mechanisms that control the apical expression of NaPi-IIa is a central issue in P_i homeostasis.

Interaction of NaPi-IIa with PDZ proteins

NaPi-IIa is an integral protein predicted to span the membrane 8–10 times, with cytoplasmic amino and carboxyl-terminal tails. In order to identify potential NaPi-IIa interacting partners, several intracellular segments of the cotransporter (amino- and carboxyl-terminal tails as well as the last intracellular loop) were first used as bait in yeast two hybrid screenings.

We found that the carboxyl-terminal tail interacts among other partners with the Na^+/H^+ exchanger regulatory factors 1 and 2 (NHERF1/EBP50 and NHERF2/E3KARP) as well as with PDZK1/CAP70/NaPi-Cap1 and PDZK2 (Fig. 1a) [11]. These last two proteins were recently renamed as NHERF3 and NHERF4, respectively. NHERF1 and NHERF2 contain two PDZ domains (post-synaptic density protein, PSD95; Drosophila disk large tumor suppressor, DlgA; *zonula* occludens-1 protein, ZO-1) and a carboxyl-terminal

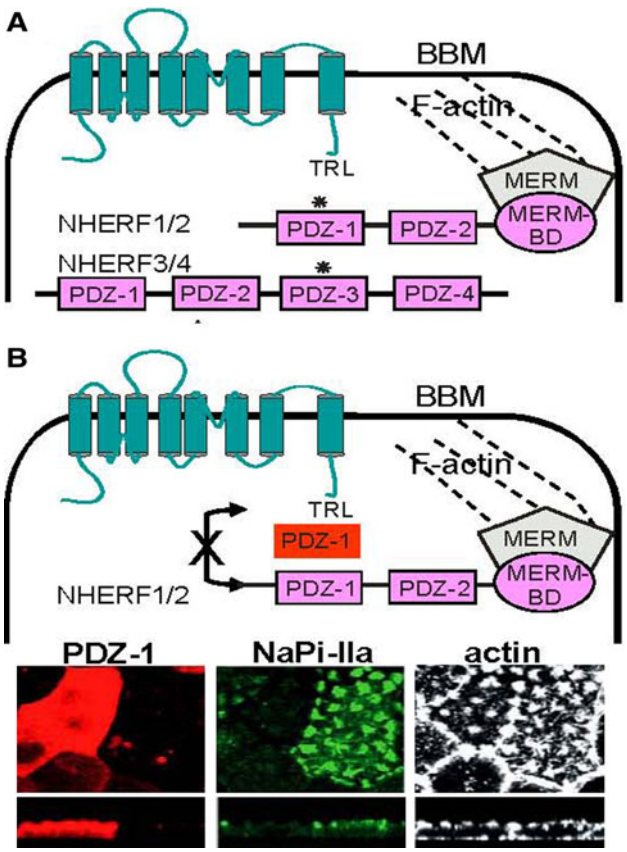

Fig. 1 NaPi-IIa interacts with the four members of the NHERF1 family. Schematic representation of NaPi-IIa and the four members of the NHERF family located on the brush border membrane (BBM) of renal epithelial cells (**a**): the interacting PDZ-domains of the NHERF proteins are indicated by asterisks. The Moesin-Ezrin-Radixin-Merlin-binding domain (MERM-BD) of NHERF1/2 interacts with the MERM family of actin-binding proteins, providing a bridge to the actin cytoskeleton. Preventing the association of NaPi-IIa with NHERF1 impairs the apical expression of NaPi-IIa (**b** modify from [13]): confocal microscopy of OK cells transfected with a plasmid encoding the myc-fused PDZ1 of NHERF1. Cultures were stained for myc-PDZ1 (*red*), the endogenous NaPi-IIa (*green*) and actin (*white*). The *patches* represent actin-enriched apical microdomains were NaPi-IIa is predominantly enriched. The transfected myc-PDZ1 has a dominant negative effect, as indicated by the observation that cells expressing myc-PDZ1 have a reduced expression of NaPi-IIa in the apical microvilli despite a normal actin cytoskeleton

Moesin-Ezrin-Radixin-Merlin (MERM) binding domain that indirectly connects with actin. NHERF3 and NHERF4 contain four PDZ domains and their binding to the actin cytoskeleton has been proposed to be mediated via association with NHERF1. Indeed, NHERF1/2 were shown to interact with the fourth PDZ domain of NHERF3 [12]. NaPi-IIa interacts specifically with the first PDZ domain of NHERF1/2 and with the third PDZ domain of NHERF3/4. In all four cases, the association is strictly dependent on the presence of the last three residues of NaPi-IIa (TRL) that therefore act as a PDZ-binding motif. In our initial screen we found that NHERF3/4 hits represented about 70% of all interacting clones, whereas NHERF1/2 hits contributed to less than 10% [11]. This observation may just reflect the different relative abundance of transcripts in the kidney cDNA library. However, in vitro analysis indicated that the interaction of NaPi-I (a cotransporter which carboxyl-terminal tail has high homology with NaPi-IIa) with NHERF3 is stronger than its association with NHERF1 [12]. The four NHERF isoforms are coexpressed with NaPi-IIa at the BBM of renal proximal tubules, but their membrane expression is not regulated by factors that modulate the apical abundance of the cotransporter.

Interaction of NaPi-IIa with NHERF1: functional effect

Early experiments showed that removal of just the carboxyl-terminal TRL residues of NaPi-IIa results in a protein that accumulates massively in the cytoplasm upon transfection in proximal epithelial cells, unlike the wild type cotransporter that is expressed preferentially at the apical membrane [16]. The finding that the three terminal residues act as a PDZ-binding motif allowed us to clarify the molecular mechanism underlying these early observations. Thus, we could show that preventing the association of NaPi-IIa with NHERF1, by transfecting OK cells only with the interacting PDZ domain of NHERF1 impairs the apical expression of the cotransporter (Fig. 1b) [13]. Together, these findings suggested that the association with NHERF1 via the terminal TRL stabilizes the expression of NaPi-IIa at the BBM. Such mechanism was confirmed when Shenolikar and colleagues published the generation of the NHERF1 knock out. They reported that the absence of NHERF1 results in a reduced expression of NaPi-IIa in the proximal BBM, leading to an increased urinary excretion of P_i and hypophosphatemia [27].

PTH is a phosphaturic hormone that acts by regulating the apical expression of NaPi-IIa. PTH binds to a G-protein coupled receptor able to activate the adenylyl cyclase (AC)/PKA as well as the phospholipase C (PLC)/PKC pathways. Both cascades lead to endocytosis and lysosomal degradation of NaPi-IIa (for review, see [4]). The AC effect seems to be solely mediated via the classical PKA pathway, without contribution of the exchange protein directly activated by cAMP (EPAC) [15]. Unlike NaPi-IIa, NHERF1 is not endocytosed upon PTH treatment [9], suggesting that the complex of both proteins must be dissociated prior to internalization of the cotransporter (Fig. 2a). Indeed the amount of NaPi-IIa that co-immunoprecipitates with NHREF is reduced upon incubation with PTH (Fig. 2b). In addition, incubation with PTH, or pharmacological activation of PKA or PKC, leads to hyperphosphorylation of NHERF (Fig. 2c), whereas NaPi-IIa does not seem to be phosphorylated either basally or after PTH treatment (Fig. 2d) [9]. Based on these observations, we proposed that phosphorylation of NHERF1 could inhibit its association with NaPi-IIa, thus rendering a cotransporter with reduced stability at the BBM. This hypothesis was later on confirmed by EJ

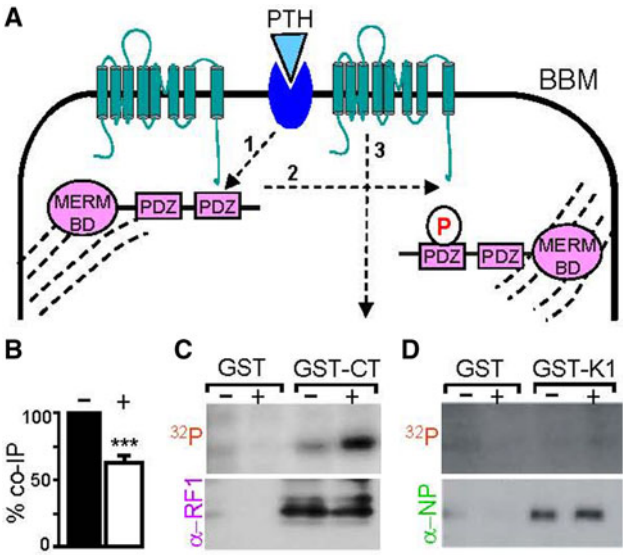

Fig. 2 PTH regulates the association of NaPi-IIa and NHERF1 and increases the phosphorylation of NHERF1. Schematic representation of the PTH-induced effects (**a**): binding of PTH to its receptor results in phosphorylation of NHERF1 (*1*), dissociation of NaPi-IIa/NHERF1 complexes (*2*) and endocytosis of NaPi-IIa (*3*). PTH treatment reduces the amount of NaPi-IIa that coimmunoprecipitates (co-IP) with NHERF1 (**b** [9]): OK cells were transfected with V5-tagged NaPi-IIa. Cultures were treated with leupeptin (to prevent lysosomal degradation) followed by incubation in the absence (−) or presence (+) of 1–34 PTH. Upon cellular lysis, NHERF1 was immunoprecipitated with an NHERF antibody, and the immunoprecipitated material was processed for Western blot with V5 and NHERF1 antibodies. *** $P \leq 0.001$. PTH treatment leads to phosphorylation of NHERF1 (**c** [9]): slices of mouse kidneys were phosphorylated in the presence of ^{32}P followed by incubation in the absence (−) or presence (+) of 1–34 PTH. NHERF1 was then pulled down using GST fused to the C-terminal tail of NaPi-I (GST-CT). Upon elution, samples were analyzed for ^{32}P incorporation (*top panels*) or processed for Western blot with NHERF1 antibodies (*bottom panel*). PTH treatment does not induce phosphorylation of NaPi-IIa (**b** [9]): kidney slices were phosphorylated in the presence of ^{32}P and leupeptin followed by incubation in the absence (−) or presence (+) of 1–34 PTH. NaPi-IIa was pulled down using GST fused to PDZK1 (GST-K1) and process as indicated above. The top panel shows the ^{32}P autoradiogram and the bottom panel a Western blot with a NaPi-IIa antibody

Weinman's group [34], who reported that PTH-induced dissociation of NaPi-IIa/NHERF1 complexes involves phosphorylation of a serine residue (S77) within the first PDZ domain of NHERF1.

In addition to its role as a scaffold for membrane proteins, NHERF1 is also known to mediate the assembly of intracellular signaling molecules (for review see [33]). Thus, NHERF1 interacts with both the PTH receptor and PLC, and it has been proposed that NHERF may control the intracellular pathway activated upon PTH binding [18]. We had previously reported that although activation of apical and basolateral PTH receptors results in internalization of NaPi-IIa they do not share similar intracellular pathways. In particular, apical receptors seem to activate preferentially the PKC dependent pathway whereas basolateral receptors signal preferentially via PKA [29]. Therefore, in collaboration with EJ Weinman's group we investigated whether the effect of 3–34 PTH, a PTH fragment that activates exclusively PKC, is preserved in the absence of NHERF1. Although 3–34 PTH downregulated NaPi-IIa in wild type mice, it failed to do so in NHERF1 knock outs [6]. This failure associated with the inability of PTH to activate PLC in the mutant mice. Since the expression of NHERF1 in the renal epithelia is restricted to the BBM, our findings are consistent with the proposal that in the presence of NHERF the PTH receptors (apical) signal via activation of the PLC/PKC pathway, whereas in the absence of NHERF these receptors (basolateral) would signal preferentially through activation of PKA [18]. Therefore, NHERF does not only control the basal expression of NaPi-IIa but also the intracellular signals that regulate its abundance.

Interaction of NaPi-IIa with NHERF3: functional effect

As mentioned above, NHERF3/4 represented about 70% of all the hits in our yeast two hybrid screen. In addition to NaPi-IIa, NHERF3 also interacts with several other transporters expressed in the proximal BBM (Na^+/H^+ exchanger NHE3, organic cation transporter OCTN1, chloride/formate exchanger CFEX and urate/anion exchanger URAT1) as well as with regulatory factors (NHERF1 or PKA-anchoring protein D-AKAP2) [12]. Due to the presence of 4 PDZ domains, NHERF3 may form a large network of membrane proteins and regulatory factors. Similar to NHERF1, the expression of NHERF3 remains unaffected upon incubation of kidney samples with PTH, suggesting that PTH also destabilizes its association with NaPi-IIa. Although NHERF3 is phosphorylated under basal conditions, PTH or independent activation of PKA or PKC do not regulate the phosphorylated status of the protein (Déliot and Hernando, unpublished observations). This suggests that NHERF3 is not a target for PTH-activated kinases and that the regulated dissociation of NaPi-IIa from NHERF3 does not depend on the same molecular mechanism that its dissociation from NHERF1.

The membrane expression of NaPi-IIa is not altered in NHERF3 deficient mice that also have normal expression of NHERF1 [5]. Regulation of NaPi-IIa by PTH or in response to pharmacological activation of PKA and PKC is also normal in these animals. Only upon challenging with diets containing high or low P_i levels we could detect subtle differences between wild type and NHERF3 deficient mice, namely downregulation of NaPi-IIa in response to high dietary P_i is more pronounced in the absence of NHERF3 [5].

Taken together, the four members of the NHERF family interact with NaPi-IIa in vitro but they do not have redundant roles in vivo. Instead, NHERF1 is specifically required for the stabilization of the cotransporter at the apical membrane and its absence is not compensated by the presence of the other isoforms in the BBM.

Interaction of NaPi-IIa with GABARAP: functional effect

More recently, in collaboration with OW Moe's group we made a new yeast two hybrid screen using as bait the whole NaPi-IIa protein just with the last terminal TRL truncated. This study identified the $GABA_A$-receptor associated protein (GABARAP) as a new interacting partner of NaPi-IIa [22]. The domain of GABARAP involved in the interaction is located between residues 36 and 68, a stretch shown previously to mediate association with several other partners, including $GABA_A$ receptors [32]. However, the domain of NaPi-IIa responsible for the interaction remains elusive, as our attempts to identify it by using discrete intracellular domains of NaPi-IIa in GST pull down assays failed.

There are a number of studies showing that GABARAP promotes the membrane expression of $GABA_A$-receptors in cell culture; however, the localization of these receptors is normal in GABARAP deficient mice [20]. We reported that these animals have a reduced urinary excretion of P_i associated with upregulation of NaPi-IIa (Fig. 3) [22]. NHERF1 is also heavily upregulated in the mutant mice. The molecular explanation for this finding remains unknown but does not seem to involve association of GABARAP with NHERF1. Despite the defect in renal handling, GABARAP deficient mice have normal levels of P_i in serum. This normophosphatemia is most probably due to a compensatory reduction of the intestinal absorption of P_i. This compensation is suggested by the finding that NaPi-IIb, the major cotransporter involved in intestinal absorption of P_i [14, 24], is downregulated in GABARAP knock outs (Fig. 3) [23]. Interestingly, also NaPi-IIb deficient mice are normophosphatemic: they seem to compensate the reduced intestinal absorption of P_i (due to the absence of NaPi-IIb) with an

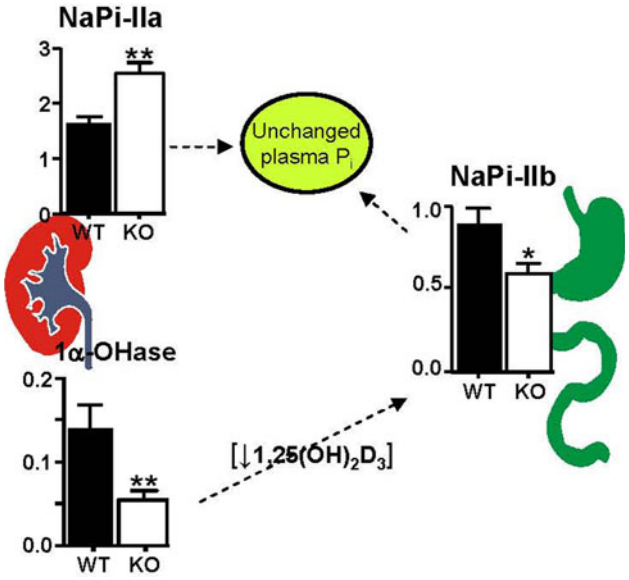

Fig. 3 Expression of Na/Pi-cotransporters in the absence of GABARAP. GABARAP deficient mice (KO) shows an increased expression of NaPi-IIa in renal BBM and a reduced expression of NaPi-IIb in BBM from ileum as compared with wild type mice (WT). The net effect of both changes probably explains the normophosphatemia in the mutant mice. Graphics show the quantification of cotransporters as their ratio to the amount of actin [22, 23]. The mRNAs levels of the 25 hydroxyvitamin D-1α hydroxylase (1α-OHase) are reduced in kidneys from GABARAP deficient mice; therefore, a low renal production of $1,25(OH)_2D_3$ could be responsible for the reduced expression of NaPi-IIb in these mice. The graphic shows the quantification of 1α-OHase mRNA normalized to the hypoxanthine–guanine phosphoribosyltransferase [23]. $*P \leq 0.05$, $**P \leq 0.01$

increased renal reabsorption (through upregulation of NaPi-IIa) [24]. Within the intestinal tract, NaPi-IIb is located preferentially in the ileum and its expression in under control of $1,25(OH)_2D_3$ (for review see [4]). The expression of the 25 hydroxyvitamin D-1α hydroxylase (1α-OHase), the enzyme that produces the active form of $1,25(OH)_2D_3$, is reduced in the kidneys from GABARAP deficient mice (Fig. 3), whereas the levels of the catabolic 24 hydroxylase are similar in both the groups [23]. These data are consistent with a reduced renal synthesis of $1,25(OH)_2D_3$ in the mutant mice which in turn lead to a reduced expression of NaPi-IIb. Interestingly, both GABARAP and NaPi-IIb deficient mice are normocalcemic; however, while urinary excretion of Ca^{++} is normal in the absence of GABARAP [23], it is increased in NaPi-IIb deficient animals [24]. There is no information regarding the presence of renal mineral deposits in these animals.

Although GABARAP was first identified as a ligand of the γ-subunit of $GABA_A$ receptors, it was later on shown to interact with several proteins involved in intracellular trafficking among them the clathrin heavy chain (for review see [19]). We have evidences that PTH-induced endocytosis of NaPi-IIa takes place at least partially via clathrin-coated pits [1], opening the question of whether the absence of GABARAP would blunt the hormonal effect. However, PTH downregulates the expression of NaPi-IIa to comparable levels in wild type and GABARAP deficient mice. Similarly, there are no differences in the adaptation of the cotransporter to acute dietary P_i changes [22].

In summary, NaPi-IIa interacts with the NHERF family of PDZ proteins as well as with GABARAP. The absence of NHERF1 results in reduced apical expression of NaPi-IIa, phosphaturia, hypophosphatemia and impaired PKC-mediated PTH regulation. In contrast, the absence of GABARAP results in increased apical expression of NaPi-IIa, and reduced urinary excretion of P_i. However, circulating levels of P_i remain normal probably due to an intestinal compensation.

Acknowledgments This work was supported by the Swiss National Science Foundation Grant 44342003 (to HM), and the Sixth European Frame Work EuReGene Project Grant 005085 (to HM). SC Reining was supported by a fellowship from the University Research Priority Program "Integrative Human Physiology", University of Zurich.

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